

# Contact allergy to propolis and beeswax

Occurrence, diagnostics, and chemistry

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UNIVERSITY OF GOTHENBURG

Gothenburg 2022

Cover illustration: Encaustic painting (in which pigments are mixed with hot, liquid beeswax) by Sylvia Windelöv, Skövde, Sweden

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ISBN 978-91-8009-929-5  
ISBN 978-91-8009-930-1

Printed in Borås, Sweden 2022  
Printed by Stema Specialtryck AB

Even the smallest steps move you forward



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### ABSTRACT

Cosmetics, including biocosmetics and “natural” skin remedies are common causes of contact dermatitis. Two frequent ingredients in biocosmetics are beeswax and propolis, which are known causes of contact allergy. These ingredients share components, with each other, and with plant-derived products and fragrances. The composition of propolis varies according to the plants growing in the vicinity of the beehive. How this variation influences the tendency of the propolis to cause contact allergy is not known.

The aim of this dissertation was to investigate contact allergy to beeswax and to propolis of different geographical origins. The method for demonstrating contact allergy is patch testing, a standardized provocation test.

We studied the frequency of contact allergy to beeswax and propolis in patients with cheilitis and facial dermatitis. Two studies regarding contact allergy to propolis of different geographical origins (from China, Lithuania, North America, and Sweden) were carried out. One included patients referred for patch testing in West Sweden, and the other included such patients in Denmark, Lithuania, and Spain. High frequencies of contact allergy to beeswax and propolis, were found. Small differences between the four types of propolis were demonstrated in each country. Propolis originating from China, and from Lithuania gave statistically higher frequencies of contact allergy than propolis from Sweden in the four countries together. High frequencies of concomitant positive patch test reactions between propolis, beeswax, and plant-related products and fragrances were found.

Beeswax and propolis should be considered as causes of contact allergy in patients with cheilitis and facial dermatitis. Patch testing with just one propolis preparation detected about half of the propolis-allergic patients. Contact allergy to propolis was so common that it should be considered for inclusion in regular patch testing.

**Keywords:** Beeswax, caffeic acid, caffeic acid 1,1-dimethylallyl ester, caffeic acid phenylethyl ester, cera alba, cera flava, cheilitis, concomitant reactivity, patch test, propolis.

ISBN 978-91-8009-929-5

ISBN 978-91-8009-930-1

# SAMMANFATTNING PÅ SVENSKA

Eksem är ett stort problem i samhället, både i arbetsliv och på fritid. Det kan orsakas av ämnen eller verksamheter som irriterar huden och också av kontaktallergi mot ämnen huden kommer i kontakt med. Väldigt många ämnen kan ge upphov till kontaktallergi. Undersökning av om det finns en kontaktallergi görs med lapptest, en standardiserad provokationstest. De vanligaste grupperna av ämnen som patienter reagerar mot är metaller, konserveringsmedel, parfymämnen och ämnen av botaniskt ursprung.

Två ämnen som kan ge upphov till kontaktallergi, men hittills inte testats rutinmässigt i Sverige, är bivax och propolis (bikitt).

Bivax är en produkt från honungsbin, som används av bina för att bygga vaxkakor, där de förvarar bland annat ägg, larver och honung. Det används i de flesta läppstift och läppcerat för sina utmärkta kosmetiska egenskaper, men har också en del industriell användning, huvudsakligen tack vare egenskapen att vara vattenresistent. Bivax kan tyvärr ge upphov till kontaktallergi, men hur vanligt detta är i Sverige är okänt, då det hittills inte är systematiskt undersökt.

Propolis är en annan produkt som tillverkas av honungsbin, och de använder det till tätning och lagning av sprickor i bikupan. Bina använder också propolis som skydd mot skadliga mikroorganismer i kupan. Traditionellt används propolis i naturmedel och naturkosmetika för sin positiva biologiska, huvudsakligen bakteriedödande effekt. Tyvärr kan även detta ge upphov till kontaktallergi, vilket är vanligt i Mellan- och Östeuropa där det undersökts. Det är okänt hur vanligt propolis är som orsak till kontaktallergi i Sverige. Eftersom bina delvis använder växtextrakt vid tillverkningen, är sammansättningen av propolis beroende på vilka växter som finns runt kupan. Om denna variation även ger variation i tendensen hos propolis från olika länder att ge upphov till kontaktallergi är inte känt.

Syftet med den här avhandlingen var att undersöka hur vanligt kontaktallergi mot bivax är bland patienter med läppeksem. Vidare att undersöka hur vanligt kontaktallergi är hos eksempatienter generellt i Sverige, och om det är några skillnader ur allergisynpunkt mellan propolis från olika geografiska områden.

Vi har undersökt kontaktallergi mot bivax hos patienter med eksem på läppar och i ansikte och där funnit hög förekomst av kontaktallergi, både mot gult och vitt vax, liksom mot propolis. Det var hög samreaktivitet ur allergisynpunkt mellan de två sorternas vax och propolis, och även gentemot parfymämnen och ämnen av botaniskt ursprung.

Lapptestning har också gjorts på patienter som remitterats på grund av misstanke om kontaktallergiskt eksem för att undersöka den totala frekvensen av propolisallergi i Västsverige och i Danmark, Litauen och Spanien. För att försöka påvisa möjliga skillnader i frekvens av kontaktallergiska reaktioner har vi testat med propolis från Kina, Nordamerika, Litauen och Sverige. Vi har funnit små, men inte statistiskt säkra skillnader mellan propolis av dessa fyra ursprung i de olika länderna. Sammantaget i alla länderna var det statistiskt signifikant mer frekvent med kontaktallergi med propolis från Kina och Litauen jämfört med propolis från Sverige. Undersökningen i Sverige pekar på att kontaktallergi mot propolis är så vanligt att det bör ingå i det standardbatteri av ämnen man rutinmässigt testar med i Sverige. Frekvensen patienter positiva för propolis var även i de övriga länderna så hög att det bör övervägas att inkludera propolis i respektive lands rutinmässiga testning. Ett viktigt fynd var att den i Sverige för lapptestning vanliga, kommersiellt tillgängliga typen av propolis – från Kina, bara påvisade drygt hälften av det totala antalet patienter som reagerade mot propolis.

Det finns undersökningar som pekar på vilka ämnen i propolis som orsakar allergi, men det är inte fullständigt utrett.

Eftersom propolis delvis är av botaniskt ursprung, liksom många parfymämnen, har också frekvenserna av samreaktioner med växtrelaterade ämnen och parfymämnen undersökts. Vi har funnit hög frekvens av sådana samreaktioner i alla länderna, liksom av samreaktioner mellan propolis och bivax. Det senare är inte förvånande då bivax är en betydande beståndsdel i propolis, och propolis ofta förekommer som förorening i bivax. Om ytterligare ämnen i bivax bidrar till förekomsten av kontaktallergi är tills vidare oklart.

### **Slutsatser**

Kontaktallergi mot bivax var i vår undersökning vanligt som orsak till eksem på läppar och i ansikte. Detta är av stor klinisk betydelse, då en vanlig reaktion när en patient får ett torrt, fjällande eksem på läpparna är att använda mer läppbalsam – som ofta innehåller bivax. Det är oklart om kontaktallergi mot bivax enbart beror på reaktion mot propolis, som förorening i bivaxet, eller mot andra, ännu inte identifierade ämnen i bivax.

Kontaktallergi mot propolis var i våra undersökningar så vanligt att man bör överväga att inkludera det i det standardbatteri av testämnen som rutinmässigt används. Vi har inte kunnat påvisa några stora skillnader i tendens att ge upphov till kontaktallergi mellan propolis av olika geografiskt ursprung. Dock skulle det vara värdefullt att påvisa något ämne som kan användas som signalämne vid misstanke om propolisallergi då många

propolisallergiska patienter missas vid test med enbart en typ. Både propolis och bivax samreagerar ofta vid lapptest med varandra och med andra testämnen av botaniskt ursprung och med parfymämnen. Detta är betydelsefullt då patienter bör informeras om att undvika även ämnen som samreagerar vid positiva testreaktioner.



# LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

- I. Nyman, G. Tang, M. Inerot, A. Osmančević, A. Malmberg, P. Hagvall, L. Contact allergy to beeswax and propolis among patients with cheilitis or facial dermatitis. *Contact Dermatitis* 2019; 81: 110-116.
- II. Nyman, G. Oldberg Wagner, S. Prystupa-Chalkidis, K. Ryberg, K. Hagvall, L. Contact Allergy in Western Sweden to Propolis of Four Different Origins. *Acta Derm Venereol* 2020; 100: adv 00256
- III. Nyman, G. Giménez-Arnau, AM. Grigatiènè, J. Malinauskienė, L. Paulsen, E. Hagvall, L. Patch Testing with Propolis of Different Geographical Origins in a Baseline Series. *Acta Derm Venereol* 2021; 101: adv00591.

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## ABBREVIATIONS

ACD	Allergic contact dermatitis
°C	degrees Celsius
CA	Contact allergy
CADE	Caffeic acid 1,1-dimethylallyl ester
CAPE	Caffeic acid phenylethyl ester
CAS	Chemical Abstracts Service
CI	Confidence interval
DPRA	Direct Peptide Reactivity Assay
ESCD	European Society of Contact Dermatitis
FM I	Fragrance mix I
FM II	Fragrance mix II
GPMT	Guinea Pig Maximization Test
h-CLAT	Human Cell Line Activation Test
HPLC	High-Performance Liquid Chromatography
IFN- $\gamma$	Interferon- $\gamma$
IL-17	Interleukin-17
INCI	International Nomenclature Cosmetic Ingredient
LC	Langerhans cell
LLNA	Local Lymph Node Assay
MPR	<i>Myroxylon pereirae</i> resin
PET	in petrolatum

T-cells	Thymus-derived lymphocytes
TNF- $\alpha$	Tumour necrosis factor- $\alpha$
ToF-SIMS	Time-of-Flight Secondary Ion Mass Spectrometry
UV	Ultraviolet

# 1 INTRODUCTION

Honey bees are fascinating insects that have been populating the world for a very long time.

Far beyond "the old Greeks", an about 50-million-years old honey bee, rather similar to a modern honey bee has been found fossilized, and the current most common genus of honey bees, *Apis*, is believed to be about 25 million years old (1).

Honey bees live in colonies in nests or hives often inhabited by 20 000 to 50 000 bees (2). There are different types of bees in a hive. The queen is the only one laying eggs, which is her main task. She is cared for, fed, and maintained by specialized worker bees. The males - the drones, don't participate in the running of the hive but are fed and maintained by worker bees. The drones are the only bees without stingers. Their only task is to fertilize the queen once during her lifetime, in a fertilizing flight, after which the drones die. Most of the inhabitants in the hive are different types of worker bees. Their tasks change over time. First, they work inside the hive, maintaining it, cleaning it, and caring for the queen, drones, eggs, and larvae. The worker bees also process collected nectar and pollen. They defend the hive and keep its inner temperature at a reasonable level, between 31 - 35°C. Later in life they turn into collecting bees and spend most of their waking time out of the hive. Honey bees are not very long-lived; a worker bee lives 4 – 6 weeks during summer, and over winter for those hatched late in autumn, while drones live a little longer, some months, and a queen one to three years.

Bees defend the hive by stinging, but seldom sting when away from the hive, if not directly attacked. A worker bee releases venom through the stinger into the wound. As the stinger is barbed the bee cannot withdraw it, so when trying to do so, the sting apparatus breaks, and the bee subsequently dies. When stinging, a bee also releases alarm pheromones which alerts other bees to attack and sting on the same site (1).

*Apis mellifera* is the most important species of honey bees to humans. It was originally native to Europe up through southern Scandinavia, in the westernmost part of Asia, and in Africa. This species has since been spread by man to most parts of the world, since they are so productive and amenable to management.

Honey is the way in which honey bees store their food. It is produced from nectar, which is collected by the bees, and is their main source of carbohydrates

(2). Throughout history, honey has to mankind been the most valuable product from bees (1). When humans started to use honey and other bee products, however, is not known. There are plenty of indications that its use goes back as long as there are any records made by humans. It has been suggested that honey hunting, when hunter/collectors gathered honey from wild bee nests, has occurred as long as man has existed (1). There are rock paintings, about 8 000 years old, in Spain showing honey hunting. Besides honey, the main product from bees, is beeswax, which has also been used in different parts of the world throughout history (1). For example beeswax was used for copper casting about 5 000 years ago by the societies east of the Mediterranean Sea.

Bees are the second most common feature found on sacred objects originating in Europe from 7 000 to 3 500 BC. Around 5 000 years ago in ancient Egypt the god Min was referred to as “master of the wild bees”. In many religions, including those of the Celts, Vikings, Jews, and Muslims, paradise was described with rivers of honey and/or mead, a drink made from honey. Honey is rich in sugars, mainly the simple sugars fructose and glucose, which together make up around 80 % of honey in approximately equal amounts, depending to some extent on the plant sources of nectar (2). Honey was the most used sweetener during early history, especially in temperate regions, while in the tropics it was more often used as a medicine. Historically, medical use of honey was both external and internal, and it was especially important as an antimicrobial. Its high sugar concentration can via osmosis dehydrate bacteria and kill them, while the acidity of honey prevents growth of microorganisms. The enzyme used by the bees in processing nectar into honey, called glucose oxidase, remains even in processed honey to some extent. It breaks down glucose in honey diluted with water to hydrogen peroxide, which is microbicidal. The first known description of medical use of honey is from an approximately 4 000-years old Sumerian clay tablet.

Besides honey, both beeswax and propolis (bee glue) have long been used by humans to maintain the skin and treat different skin disorders.

## 1.1 THE SKIN

The human skin is built up by three layers, called (from the outside inwards) the **epidermis**, the **dermis**, and the **subcutis** (3, 4). The epidermis consists mainly of three types of cells; keratinocytes, important for the skin barrier and potentiation of immunological response, melanocytes, responsible for pigment production of the skin, and Langerhans cells (LC), which are antigen-presenting. The outermost part of the epidermis is the stratum corneum, which consists of flattened keratinocytes and is an important barrier to external substances. The dermis consists mainly of fibroblasts, producing the fibers – collagen, important for tensile strength, and elastin, which are important for the elasticity of the skin. Fibroblasts also produce the basic substance, that binds water. Furthermore, the dermis contains mast cells, important to the immunological defence, as well as blood and lymph vessels and nerves. The subcutis layer contains lipocytes, which store subcutaneous fat, which in turn protects against trauma and cold.

Dermatitis, or eczema, is an inflammatory condition of the two outer layers of the skin. It includes one or more of the following symptoms: erythema, infiltration, scaling, vesicles, papules and itching (5).

## 1.2 CONTACT DERMATITIS

One common type of eczema is contact dermatitis (CD), caused by a physical or chemical contact with a causing agent or activity (6). There are two main types of CD: - irritant, which is by far the most frequent, and allergic. Irritant CD is caused by external factors that disturb the epidermis' protective function of the skin, such as chemicals, low humidity, heat, cold, ultraviolet (UV) irradiation, or water, but also mechanical factors like friction or occlusion (7, 8). Irritant chemicals cause a non-specific dermatitis via release of pro-inflammatory cytokines, mainly from keratinocytes. Cytokines are small, soluble proteins important for cell signalling, secreted by inflammatory cells (9).

Contact allergy (CA) is caused by an immunological reaction, sensitization, to a foreign chemical in contact with the skin (6). When an individual has been sensitized to a chemical, a renewed contact with this chemical can result in an allergic CD (ACD). CA is not a disease, but rather the immunological response to sensitization. On the other hand ACD is a disease that occurs when an individual with CA to a chemical comes in contact with that chemical, and this cause a dermatitis (10). The risk of becoming sensitized is dependent on a

chemical's capability to sensitize, the concentration of the chemical, the duration of contact, and the capability for a substance to penetrate the skin. The penetration increases when the skin barrier is damaged, for example from an irritant dermatitis (8). Around 15 – 30 % of the population in the western world are sensitized to at least one chemical (11-13). The most frequent cause is nickel (around 11 %), but also other metals, as well as fragrances and preservatives, frequently cause CA. CA is about twice as common in females than in males (11). Occupational CD is a special problem and regarded as the most common occupational disease in many countries. Precise incident rates for occupational CD are difficult to calculate and compare, however, because of different regulations, registration systems, and compensation criteria in different countries (14). ACD can appear anywhere on the body, but the hands are most often affected, as usually the hands are the body parts most exposed to external factors (10).

Another type of eczema is atopic dermatitis, which is multifactorial, and often presents with dry, itchy skin (15). The question of the increased, or decreased, risk for CA in atopic dermatitis has been an issue of discussion for a long time, but it seems that atopic dermatitis is of minor importance in this context (16, 17).

### 1.3 CONTACT ALLERGY AND IMMUNOLOGY IN ALLERGIC CONTACT DERMATITIS

As the skin is the barrier of the body towards the environment, it is confronted with potentially harmful external chemicals and microorganisms. It is crucial for the body to have a system of protection against this exposure – the immune system. There are two parts of the immune system, the innate and the adaptive systems (18). The innate, general, system responds quickly, and in the same way to all foreign attacks from microorganisms or other substances. The adaptive system is a cell-mediated, second line of defense (18). It is slower, but more specific than the innate system, and includes cells that can be activated by antigens. Thymus-derived lymphocytes (T-cells) are mediators of the cell-mediated immunity. This is a delayed hypersensitivity, classified as type IV of the Gell-Coombs types of immunologic reactions (19). LC are the most important antigen presenting cells in the epidermis and form an immunological network in the skin (20).

### 1.3.1 SENSITIZATION

A hapten is a chemical that can bind to a protein and form a hapten-protein complex. Haptens are generally small, with molecular weight below about 1000 Dalton, chemically reactive, and lipid-soluble (10, 21). Haptens are often electrophilic and can form covalent bonds with nucleophilic amino acids in proteins, usually lysine or cysteine. The ability of a hapten to induce a CA depends on many factors, such as the chemical sensitization potential, how reactive the hapten is chemically, and also its capability to penetrate the skin (10). The latter can be increased if the skin is damaged or irritated and depends on the concentration of the chemical and the duration and frequency of contact. Hereditary factors may also play a role (6). Some chemicals are not reactive enough themselves to be haptens, but require activation, like oxidation, either via contact with air, or metabolically (22). These chemicals are called “prohaptens” if the reaction takes place metabolically in the skin and “prehaptens” if the chemical reaction take place before contact with the skin (23). Sensitization starts with skin contact with a hapten and ends when the individual is sensitized. This phase is also called the induction phase, and takes from a few days, to more often up to a few weeks (6). The hapten-protein complex is transported by LC via lymphatic vessels to local lymph nodes, and presented to a specific type of lymphocytes, called naïve T-cells, which become transformed to sensitized T-cells (memory cells) (6, 12). The memory cells are transported back to the skin and circulate in blood and lymph vessels, without causing any reaction, unless they are exposed to the chemical to which they were sensitized (6). A memory cell is specific to one or a few haptens, and ACD is dependent on the activation of hapten-specific T-cells. Such T-cells act as an immunological memory and can recognize a hapten if it comes in contact with the skin again. Depending on how reactive the hapten is the sensitization can be fast, sometimes after just one contact, but more often after repeated contacts, even during several years.

### 1.3.2 EFFECTOR PHASE

Following a new contact with the hapten, the specific memory cells proliferate several-fold and initiate a cascade of release of proinflammatory cytokines, mainly interferon- $\gamma$  (IFN- $\gamma$ ), tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interleukin-17 (IL-17). Chemokines, a special type of cytokines, start an immunological reaction via directing the migration of white blood cells to the site of contact, giving rise to inflammation and resulting in a dermatitis (6, 10). This is called the effector, or elicitation phase, and occurs over a few days (8-10).

## 1.4 TREATMENT OF CONTACT DERMATITIS

The overall goal in caring for patients with CD is to increase their wellbeing. The most important issue regarding ACD is prevention (24). Primary prevention, to avoid sickness, is a task for the society at large by reducing or prohibiting substances causing CA. Secondary prevention, to avoid getting symptoms, when a CA is discovered, involves informing the patient on ways to avoid contact with the causing agent(s). Tertiary prevention is treatment to reduce symptoms. This can be done by enhancing the skin barrier, or treating the dermatitis, usually with topical corticosteroids, and in some severe conditions, corticosteroids are systemically used. Regarding ACD, the most important consideration is to avoid the chemical that cause the allergy, which makes the diagnosis of the CA of utmost importance.

## 1.5 DIAGNOSIS OF CONTACT ALLERGY

Patch testing is the golden standard for diagnosing CA and has been used since the end of the 19th century (11). It can be described as a standardized provocation test. A dose of 15 – 20 mg of a suspected hapten in petrolatum (pet) is placed on the skin under occlusion in a chamber of plastic or aluminium, normally on the back, for 48 hours, and then removed (25). On day (D)3/4 and D7 after application, the area is assessed for eczema reactions. Those are graded as positive mild (+), strong (++), extreme (+++), or negative (-). They can also be doubtful (?) or irritant (IR), which are also considered negative. The boundaries between the different gradations are not very sharp. Measures have been taken to standardize patch test reading, but a certain degree of subjectivity remains, depending on the reader (26).

There are several other factors beyond the subjectivity in reading a patch test reaction which can influence the result of the patch test. Some such factors regard the test preparation, such as choice of vehicle, the concentration of hapten, variability in allergen composition, and variability between batches during the production. Conditions during transport and storage of the test preparation can also influence the patch test result.

When investigating CA, a baseline series that includes the most frequent haptens and allergens is used. Different baseline series can vary regionally according to the chemicals used in products in the country. The groups of haptens most frequently resulting in positive patch test reactions are metals, preservatives, and fragrances (13). Several of the patch test preparations from the baseline series are of botanical origin or fragrances (11, 27).

## 1.6 NATURAL PRODUCTS IN THE BASELINE SERIES

The use of natural products containing botanical extracts have increased lately, partly due to the perception that they are safer than synthetic ones (22). Some of those have a proven effect, though the positive effect of many products has not been documented. Several natural products are used both in medicinal products, and in products for personal care (23). Colophonium is one such, which is also used in glues, adhesives, and paper, as well as in rubber and in electronic industries. It causes CA rather frequent and had in Sweden a prevalence of CA of 3.5 % 2010-2017 (24). Balsam of Peru (*Myroxylon pereirae* resin, MPR), derived from a South American tree's bark is an aromatic substance used for fragrance which during the years 2010 - 2017 had a prevalence of CA in Sweden of 6.9 %. Both those are present in baseline series from different countries, and so are Fragrance mix I (FM I) and II (FM II), mixes of several fragrances, known to be able to cause contact allergy (24). Besides there are lots of different plant extracts, used in cosmetics. Sesquiterpene lactone mix, from the plant family Asteraceae, and lichen acid mix from lichens are two other plant-derived patch test preparations.

## 1.7 CONCOMITANT REACTIONS

Concomitant reactions are when a patient reacts to different haptens simultaneously (32, 33). This can be by coincidence but can also have several other explanations. Concomitant reactions can be true cross reactions, when two haptens are so closely chemically related that the immune system cannot distinguish between them; pseudo-cross-reactions, when two products contain the same hapten; chemical oxidation or metabolization of one substance to another in a product; or co-sensitization, when a patient is sensitized to two haptens at the same time. Previous studies have shown varying frequencies of concomitant patch test reactions between propolis and MPR of between 13 – 90 % (33, 34). Concomitant reactions between propolis and FM I have been found between 11 - 25 % of patients (30, 34), and between propolis and colophonium in 13 – 27 % of patients (30, 34).

## 1.8 CLINICAL RELEVANCE

In the field of ACD assessing clinical relevance is an important - and difficult – issue (35). It is normally graded as current, earlier, or unknown relevance. To be considered a current relevance, there should be a CA, exposure to the hapten in temporal connection, and symptoms in accordance with the exposure. One problem is that sometimes neither the doctor nor the patient knows all possible exposures to a hapten. In the case of a concomitant reaction, there is also the possibility that the patient has been in contact with a co-reacting hapten and never with the hapten giving the positive patch test reaction. In this case, the frequency of relevance will be falsely low.

## 1.9 INVESTIGATING SENSITISATION POTENCY

Animal tests were previously the golden standard for evaluating a compound's tendency to cause CA – for example the Guinea Pig Maximization Test (GPMT). In the GPMT, a suspected sensitizer is injected intradermally on the back of a guinea pig, and a week later the same substance is applied topically to the same area under occlusion. Two weeks after this, a patch test is performed on the flank and assessed (36, 37).

Another animal model is the Local Lymph Node Assay (LLNA). This assay uses a mouse model based on the induction phase during skin sensitization. The ears of a mouse are exposed to a possible allergen and after an induction time, the local lymph nodes are excised and the lymphocyte proliferation is measured as a measure of sensitization potency (38). This method had benefits including higher reproducibility, as well as a possibility to quantify the sensitization potency of tested compounds and to determine a safe exposure level (39).

In order to reduce animal tests, both from an ethical, and from practical and economical aspects, *in vitro* methods for evaluating the tendency of a chemical to induce CA have been developed (39). One such method, without using animals, of investigating a suspected hapten's chemical reactivity, and ability to bind to a protein covalently is the so-called “Direct Peptide Reactivity Assay” (DPRA). In this method, peptides, containing cysteine or lysine, are exposed to a suspected hapten and the depletion of peptides over time serves as a measure of reactivity of the hapten. The faster the peptide is depleted the more reactive – and allergenic - is the hapten. The method has been validated

against the animal tests, LLNA and GPMT, which have been used earlier (40). DPRA is recommended by the European Union for assessment of sensitizing potency, and should, according to the European Union, be used before experiments on animals (41).

There are also cell-based methods for investigating sensitization potency, like the “KeratinoSens” assay (42), “Human Cell Line Activation Test” (h-CLAT) (43), and the lymphocyte transformation test, which all measure the production of cytokines as a measure of sensitizing potency (44).

## 1.10 CHEMICAL ANALYSIS

A problem in analyzing complex products, like beeswax or propolis, is to extract the hapten from a product, and to do so without changing the hapten chemically or affecting the sample, which can happen when using high-performance liquid chromatography (HPLC) or mass spectrometry.

One method to detect if a suspected chemical is present in a product is the Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) (45). This is a method that can detect different ions on the surface of a product sample, without changing or affecting the sample. Ions are sent to collide with the surface of a sample, and secondary ions knocked off are accelerated towards a detector, and their relative mass is accurately determined by the time of flight to the detector.

## 1.11 ALLERGIC CONTACT FACIAL DERMATITIS AND CHEILITIS

Facial CD is frequent, and the face is the second most common site, after the hands, affected in patients referred to dermatology clinics for patch testing (46). Patients with facial dermatitis experience this as disturbing and seek medical attention earlier than patients with dermatitis on other sites (46, 47). A special type of facial dermatitis is cheilitis, which is dermatitis of the lips (Figure 1).



*Figure 1. Eczematous cheilitis, photo taken by the author*

It can be caused by CA, but also by irritation, dehydration, and UV radiation. Lipsticks and lip balms are common causes of CA cheilitis (48). A tendency to dry lips in patients with atopic dermatitis could be a possible risk factor for CA cheilitis, but this has not been clearly demonstrated (16, 17, 49). In lipsticks CA can be caused among other things by dyes, fragrances, sunscreens, and metal salts. Eczematous cheilitis is more frequent in females than in males. (50, 51). In studies of patch testing, CA have been found in 25 – 85 % of patients referred for eczematous cheilitis, (50-53). The most frequent reactions vary between different countries, but reactions to FM I, MPR, ricinoleic acid (anti-inflammatory and moisturizing agent from castor oil), dodecyl gallate (antioxidant, used as preservative), nickel, cobalt, and gold are frequent. The same substances that mostly cause allergic cheilitis are also frequently positive in patch testing of facial dermatitis, but in this group also positive test reactions to preservatives are frequent (46).

## 1.12 BEESWAX

Beeswax is produced by honeybees in glands on the ventral part of the abdomen and consists of mainly esters, fatty acids, and carbohydrates (54). These substances are seldom allergenic, but beeswax also contains small amounts of several other substances, of which some are not identified. In its natural form, beeswax is yellow (*cera flava*) but it is often purified or bleached before use into white (*cera alba*). The bees use it to build honeycombs with cells strong enough to support storage of honey and other products, as well as larval and pupal protection within the beehive (1, 55).

The quality of beeswax has been an issue due to adulteration, and during the second half of the 20<sup>th</sup> century, standards and specifications for beeswax offered for sale have been defined.

Due to its low melting point, beeswax has good cosmetic properties and has since ancient times been used in lip balm, hair wax, and in “natural” skin remedies. Its plasticity also makes beeswax good for making candles, as well as for modelling and metal casting. It is further valuable as it is impervious to water and water solutions, and relatively inert, so it can be used as a barrier coating and as a protection against water and water solutions (1). Other uses include as a water repellent on the surface of stone and cement, in impregnation of cloth and leather, and as a surface layer on sweets. Beeswax can also be used as a material for artists (Figure 2).



*Figure 2. The Honeycomb Vase. Artist: Tomáš Libertíny. Year: 2006. Permanent Collection of Cincinnati Art Museum. Photo © Studio Libertíny. By permission of the artist.*

In order to have an international uniform nomenclature and definition of constituent substances in cosmetic and topical products, there are two systems: International Nomenclature Cosmetic Ingredient (INCI) names and Chemical Abstracts Service (CAS) numbers (56). Beeswax is an INCI name with CAS numbers 8006-40-4 and 8012-89-3, and the latter is also the number for the INCI name “cera alba”, whereas “cera flava” has no INCI name.

### 1.13 PROPOLIS

Propolis is a lipophilic material produced by honey bees by mixing resinous material from plants in the vicinity of the hive with beeswax and saliva (57). In northern and central Europe resinous materials are mainly from poplars. Raw propolis (Figure 3) differs in composition, due to the vegetation where the bees are collecting the resinous exudate from the plants, but contains in general about 50 % resin, 30 % beeswax, 10 % essential oils and 5 % each of pollen and other organic compounds (58).



*Figure 3. Raw propolis. By permission of the photographer.*

Over 300 chemicals have been identified in propolis (59). Flavonoids make up the largest group of components found in propolis, though many are not exactly the same as those found in plant buds, from which material to propolis is collected by the bees (58). This is presumably due to transformation of the plant bud flavonoids by enzymes in the bee's saliva. The bees use propolis to glue and seal cracks in the beehive (60). Due to the antimicrobial effect of propolis, it is also used as a protective material guarding the colony against microorganisms, for example by covering the

walls of the entrance of the hive, and by embalming killed intruders. Propolis is popular in “natural” remedies and cosmetics and appears as an impurity in beeswax (34, 61). Propolis can also be used for artistic purposes (Figure 4).



*Figure 4. When heated, propolis looks like black glass and can be used for making art. Made by Marlene Huissoud, Paris. By permission of the artist.*

There are several records of the beneficial effects of propolis, e.g.; antimicrobial, anti-tumour, antioxidative, anti-inflammatory, and antihepatotoxic (59). As for other bee products, the vast majority of those investigations involve *in vitro* or animal tests on propolis activity against bacteria, viruses, and fungi. (62). Some investigations showing positive effects of propolis in humans have also been performed, both topically and orally, regarding for example diabetic ulcers, other wounds, and metabolic diseases (63-65). Propolis has an antibacterial effect, mainly against Gram-positive bacteria, and it has been shown that the effect differs between propolis from different geographical regions (66). Different substances are responsible for the antibacterial properties of propolis from different geographical areas (67). However, even though propolis differs chemically between different regions, they all have an antibacterial effect to some extent. This indicates that the antibacterial property of propolis is not attributable to just a few ingredients, but rather to that it is its complex composition that results in this effect. Many of the beneficial effects of propolis are attributed to flavonoids and phenolic esters (68). Caffeic acid esters (caffeates) are important anti-bacterial compounds in propolis, among them caffeic acid 1,1-dimethylallyl ester (CADE) (69).

Propolis has several INCI names: Propolis cera, propolis extract, and propolis wax, all with CAS number 85665-41-4, and propolis wax has also CAS number 9009-62-5 (56).

## 1.14 CONTACT ALLERGY TO PROPOLIS AND BEESWAX

Beeswax can cause CA, both from medical products and cosmetic allergy in for example cheilitis (52, 70, 71). Occupational CA to beeswax has also been described in an artist (72). Although beeswax is very common in cosmetic products, CA to beeswax is rarely systematically investigated. There is one investigation that found a frequency of CA to beeswax of 0.45 % in a general test population (34). In this study in 10 centers in the United Kingdom 2 828 consecutive patients were patch tested to white and yellow beeswax “as is”, and to propolis 10 % pet. The researchers found that 1.9 % were patch test-positive to propolis and 0.45 % to beeswax (ten to white and three to yellow). Only four of the beeswax-positive patients were positive also to propolis and only one of the beeswax-positive patients was positive to both white and yellow wax. To the best of my knowledge, it is not further investigated if there are differences in frequency in CA between yellow and white beeswax. Beeswax have previously been patch tested in different concentrations, 20 % (72) 30 % (52, 71, 73) and in 100 % (34, 47, 72).

Propolis is well a known cause of CA (61, 74, 75), and was first described in a beekeeper’s journal by the affected beekeeper in 1915 (76). A case report of systemic CD after ingestion of a propolis solution has been published (77), as well as a case of airborne CD (78). To my knowledge there is just one report from Slovakia of the prevalence of CA to propolis in non-dermatitis patients (79). In this study, one group of general hospital patients and one group of healthy volunteers were patch tested with 5 % propolis in ethanol, showing 1.2 – 3.3 % and 0.64 % positive results respectively. Furthermore an almost threefold rise in prevalence of positive propolis reactions were found in this study during the seven years of testing. Reports of prevalence or incidence of CA in the general population are scarce overall, though CA and ACD are frequently studied. The closest estimates, regarding propolis, although these should be made with great care, regarding propolis might be to compare with frequencies of CA to test substances that often have concomitant reactions with propolis, namely colophonium, MPR, and FM I. In studies of the general population in European countries, prevalences of 0.9 - 1.3 % (colophonium), 0.7 - 1.8 % (MPR) and 0.9 - 3.5 % (FM I) have been found (11, 80). CA to propolis in patients referred to patch test clinics ranges between 0.5 – 7.6 % (61, 74, 75), and seems to increase over time (61, 79, 81, 82). This is excluding one Polish study that differed substantially with a frequency of 15 % (75). Due to the high frequency of CA to propolis, it is included in several countries’ national baseline series of haptens, and since 2019 in the European baseline series (83, 84). The most common concentration of propolis for patch testing

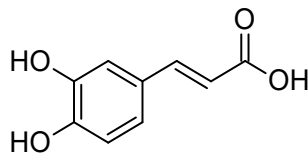
is 10 %, but some investigators have also tested with 5 % (75), 20 % (61, 75, 85), and “as is” (52).

The frequency of CA to propolis in Sweden has never been investigated. Occupational ACD due to propolis occurs mostly in beekeepers, but also in violin makers, and - players (86, 87). Besides CD, also symptoms of a Coombs’ type I – allergy to propolis can occur, like urticaria, difficulties to swallow and dyspnea, especially in beekeepers (88). Hand eczema is the most common CD in beekeepers due to propolis. However, hand eczema is more frequent in beekeepers than in the general population also except CA to propolis (89). In a study of 772 Dutch beekeepers, 158 (20 %) had hand eczema. Of the 28 of those who were patch tested with propolis eight (29 %) showed a positive test reaction to propolis (89).

Both beeswax and propolis are complex natural products, and as such not the same from one batch to another (90). Primary bee products are synthesized by the bees, while secondary products are processed by the bees from plant extracts altered to fit the utilization of the bees. Beeswax, as being a primary bee product, varies between batches less than propolis, a secondary product, as the latter’s contents to a high extent depends on the plants around the hive. This variation can be a problem regarding reproducibility of patch testing, and even if the main botanical source of propolis in temperate zones of the world is poplar, the chemical content and also the microbial activity, can differ according to the poplar species and clones (91). If there is a difference due to the geographical origin in tendency to cause CA is to the best of my knowledge just investigated once, and only with propolis from different regions in the United Kingdom (92).

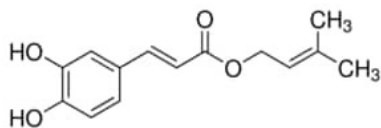
Efforts have been made to identify responsible haptens for CA to propolis. The main suspects, at least in the poplar-type of propolis, have been considered to be caffeic acid, CAS number 331-39-5 (Figure 5),

*Figure 5.*



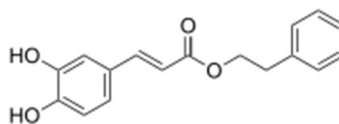
Caffeic acid

and its esters, CADE, CAS number 118971-61-2 (Figure 6), and caffeic acid phenylethyl ester (CAPE), CAS number 104594-70-9 (Figure 7) (93-95).



Caffeic acid 1,1-dimethylallyl ester

Figure 6



Caffeic acid phenylethyl ester

Figure 7

Both experimental tests on guinea pigs and patch tests on patients have demonstrated the strong sensitizing potential of these caffeates, but not of caffeic acid (93). These caffeates have also been detected in poplar bud exudates, one main component of propolis in the temperate zones of the world, and also itself sensitizing. The content of these two caffeates – and other components of propolis, can vary considerably between different batches of propolis (93). This variation depends on locality of the beehive, but also on the species or clone of poplar growing within flight range of the hive. In non-poplar propolis, other haptens than caffeates are proposed to be more important (95). Many hapten candidates have been found among the more than 300 chemicals detected, and among them benzyl salicylate has been shown to be a moderate sensitizer (93, 95). The contents of CAPE and CADE, as well as of flavonoids, are suggested to be markers of propolis safety and quality (68). These esters, claimed to be the strongest haptens in propolis, do not react easily in a non-oxidized form. They are, however, shown to easily oxidize in the presence of the enzyme tyrosinase, and the orthoquinones of the esters formed is much more reactive (22, 96). Tyrosinase is present in the skin, and the two caffeates could thus be pro-haptens. Investigations have shown that caffeic acid can oxidize to an orthoquinone (97) and that CAPE readily is oxidized to quinones, and thus more sensitizing (98).

Promising attempts have been made trying to reduce the sensitization to propolis without losing positive effects via biotransformation of the caffeates by *Lactobacillus helveticus* or *Lactobacillus plantarum* (99, 100). This is possible because many beneficial effects are depending on substances other than the suspected sensitizing culprits. For example, much of the beneficial antimicrobial effects are due to flavonoids, while the caffeates are the substances most suspected to cause sensitization. The latter are also considered important for the beneficial antioxidant effects of propolis, so there is no strict boarder between beneficial and harmful substances (67).

As several plant-related products and fragrances share the same components, or have ingredients closely chemically related to those of propolis and

beeswax, concomitant patch test reactions are common. Propolis share at least eight components with MPR, among them cinnamic alcohol, cinnamic acid, benzoic acid, and benzyl alcohol. Cinnamic alcohol is also present in FM I. Colophonium, and FM I share phenols both with propolis and MPR (94, 101, 102). Concomitant reactions between propolis, beeswax, colophonium, MPR, and FM I were detected in previous studies (34).

## 2 AIMS

The overall aim with this dissertation was to investigate CA to beeswax and propolis in order to take better care of and give better information to patients with such allergy.

More specifically, the project aims to investigate the nature of CA to the honey bees' products, beeswax and propolis, by:

- Investigating the frequency of CA to beeswax and propolis in patients with cheilitis.
- Investigating if previously suspected caffeates were important haptens in beeswax-allergic patients.
- Investigating the frequency of CA to propolis among eczema patients in West Sweden.
- Investigating the frequency of CA to propolis among CD patients in three European countries.
- Investigating possible differences in frequencies in CA to propolis of different geographical origins in Sweden and in three different European countries.
- Investigating the prevalence of concomitant test reactions between propolis/beeswax and some related substances of botanical origin and fragrances from the baseline series.

## 3 PATIENTS AND METHODS

### 3.1 SUBJECTS

Paper I: The cohort consisted of patients referred for patch testing with inclusion criteria dermatitis on the lips or face or anamnestic suspicion of CA to beeswax. Exclusion criterion was age below 18 years. Testing was performed from August 2009 until October 2016, in the dermatology department of Sahlgrenska University Hospital, Gothenburg, Sweden and in a private dermatology clinic (“Hudläkarmottagningen Telegrafan”) in Borås, Sweden. Test material was the Swedish baseline series, yellow beeswax, white beeswax (from 2013, when it was supplied), and propolis.

Further testing with caffeic acid, CAPE and CADE was offered to the 17 patients who tested positive to beeswax. Ten patients accepted and were tested with dilution series of the three chemicals. Caffeic acid was tested in 5.0 %, 0.5 %, 0.05 %, 0.005 % and 0.0005 % pet, while CAPE and CADE were both tested in 1.0 %, 0.1 %, 0.01 %, 0.001 % and 0.0001 % pet.

Paper II: Consecutive patients, 18 years or older, referred for patch testing to the dermatology departments of the four hospitals in Region Västra Götaland (West Sweden), were during a 12-month period, 2016 until 2017, tested with the Swedish baseline series and also with propolis of four different geographical origins, namely from China, North America, Lithuania, and Sweden.

Paper III: Consecutive patients, 18 years or older, referred for patch testing to the dermatology departments in Odense, Denmark; Barcelona, Spain; and Vilnius, Lithuania, and the allergology department in Vilnius, Lithuania, were patch tested during a 12-month period at each center, autumn 2017 until spring 2019. Besides testing with each respective country’s baseline series, the patients were also tested with propolis of the same batches of different geographical origins as used in paper II.

## 3.2 CHEMICALS AND PATCH TEST PREPARATIONS

Paper I: Yellow beeswax was provided by Apoteket AB (Stockholm, Sweden). White beeswax was acquired in 2013 from ACO Hud Nordic (Upplands Väsby, Sweden). The preparations in the Swedish baseline series, and propolis 10 % pet were purchased from Chemotechnique Diagnostics (Vellinge, Sweden). Caffeic acid, CAPE, and CADE were purchased from Sigma-Aldrich Sweden (Stockholm, Sweden). Nuclear magnetic resonance spectrometry was used to determine the purity levels of caffeic acid and its two esters; no impurities could be detected.

Paper II and paper III: The propolis 10 % of Chinese origin was bought from Chemotechnique Diagnostics and the propolis of North American origin was purchased from Smart Practice (Phoenix, Arizona, USA). Propolis of Swedish origin was collected by a beekeeper on the west coast in the Halland region, and propolis originating from Lithuania was collected by a beekeeper in the Kaunas region. Both the Swedish and the Lithuanian propolis were prepared into 10 % by Chemotechnique Diagnostics using the same procedure as for their regular propolis preparation. All propolis preparations were tested pet. The baseline preparations in Lithuania were bought from Chemotechnique Diagnostics, in Spain from Allergeaze Marti Tor Allergias (Barcelona, Spain), and in Denmark the baseline series were purchased from Chemotechnique Diagnostics, and Smart Practice, and included True Test (Smart Practice).

## 3.3 PATCH TESTING

Paper I: Patch test preparations of 20 mg were applied in small Finn Chambers (diameter of 8 mm; inner area of 0.5 cm<sup>2</sup>; Eptest, Tuusula, Finland) on Scanpor tape (Norgesplaster, Vennessla, Norway) to each patient's back, left occluded for 2 days, and then removed by the patient. The amounts used for patch testing were 75 mg for yellow beeswax and 79 mg for white beeswax, both "as is". According to recommendations by the European Society of Contact Dermatitis (ESCD), readings were performed on D3/D4 and D7, and positive reactions were graded by a dermatologist following standard procedures (25). Clinical relevance was assessed based on patient history and time-related exposure to products containing beeswax. Clinical relevance was rated as current, previous, or unknown.

Paper II: Patch testing with the Swedish baseline series was carried out according to the producer's instructions. Eight-mm Finn Chambers on Scanpor tape were used in all centers, except Gothenburg, which used IQ Ultra chambers (8×8 mm; Chemotechnique Diagnostics). A 20-mg dose of propolis was applied with the Finn Chamber and 25 mg with the IQ Ultra chamber. Patch-testing and reading of the patient's results on D3/4 and D7 were carried out according to ESCD guidelines (25). Relevance was assessed based on patient history of time-related exposure to products containing propolis.

Paper III: The respective baseline series was applied according to the normal routines in the three countries, respectively. In all centers, a dose of 20 mg of propolis was applied in 8-mm Finn Chambers on Scanpor tape. Reading of the patients' results was performed according to the guidelines from the ESCD. Assessment of relevance was based on patient history regarding time-related exposure to propolis.

### 3.4 TIME-OF-FLIGHT - SECONDARY ION MASS SPECTROMETRY

Samples of yellow and white beeswax were examined regarding surface haptens (caffeic acid, CAPE, and CADE) with ToF-SIMS. The method used to record ToF-SIMS spectra of negative ions with a mass resolution of  $m/\Delta m = 8\,000$  full widths at half maximum at  $m/z$  500, was with a ToF-SIMS 5 instrument (IONTOF, Münster, Germany) equipped with a liquid metal primary ion source. To analyze the ToF-SIMS data, the software SURFACE LAB (version 6.3; IONTOF) was used. Signals for  $[C]^-$ ,  $[CH]^-$ ,  $[C2]^+$ ,  $[C3]^+$  and  $[C16H31O2]^+$  were used for calibration.

### 3.5 STATISTICS

Paper I: Fisher's exact test was used to compare frequencies of positive test reactions between the groups with and without positive tests for beeswax.  $P < 0.05$  was considered as significant and all tests were two-sided. OPENEPI (<http://openepi.com>) was used for calculation of confidence intervals (CI).

Paper II: Fisher's exact test was used to compare proportions of patients positive to two types of propolis of different origins. The exact binomial test was used for paired tests of differences between patients being positive for propolis and MPR, colophonium, and FM I. All tests were two-sided, and  $P < 0.05$  were considered significant. CIs were calculated using OPENEPI (<http://openepi.com>).

Paper III: Fisher's exact test was used to compare proportions of patients positive to two types of propolis of different origins. It was also used to compare proportions of patients positive to propolis in the different countries where they were tested. The exact binomial test was used for paired tests of differences between patients being positive for propolis and MPR, colophonium, and FM I. All tests were two-sided, and  $P < 0.05$  were considered significant. CIs were calculated using OPENEPI (<http://openepi.com>).

Power was calculated using (<http://powerandsamplesize.com>) comparing differences in patch-test-positive patients to two different origins of propolis in all four included countries.

### 3.6 ETHICS

Ethical approval was obtained from the local ethical committee for studies I, reference number 409-16, and II, reference number 721-16.

For study III, ethical approval from the local ethical committee was obtained in Lithuania, whereas in Denmark and Spain the testing was performed according to current local ethical standards and to national regulations.

## 4 RESULTS

**Paper I:** Out of 95 patients patch tested for cheilitis, eczema of the face, or suspicion of contact allergy to beeswax, 18 % in total were positive to beeswax. Furthermore, 17 % were positive to yellow beeswax and 18 %, of 49, tested positive to the white beeswax (Table 1). Of the 49 tested against both yellow and white beeswax, five were positive to only yellow and one to only white. A total of 84 patients were also tested to propolis and 19 % of those tested positive. Three propolis-negative patients were positive to beeswax. Current relevance was assessed in 16 of the 17 positive patients (94 %).

*Table 1: Positive patch test reactions to white or yellow beeswax or propolis among patients with contact cheilitis, facial dermatitis or suspicion of beeswax allergy.*

	Patients tested (number)	Patients with positive test result (number)	Percentage of patients with positive test result	CI
Cera flava or cera alba	95	17	18	11 - 28
Cera flava	95	16	17	10 - 27
Cera alba	49	9	18	9 - 24
Propolis	84	16	19	11 - 30

Abbreviations: CI, confidence interval

Among the patient's own products, the main culprit in the beeswax-positive patients was a lip balm called "Försvarets hudsalva", or similar products, which nine of the 17 positive patients had used. This product contains yellow beeswax.

Concomitant reactions among the patients of the cohort to allergens in the baseline series related to plants and fragrances were common, mainly to MPR (15 %), FM I (13 %), and colophonium (10 %) (Table 2). These observed frequencies are significantly higher than in an unselected test population,

during 2015, from Gothenburg, Sweden and about three, two and five times as frequent, respectively (Table 2).

*Table 2: Differences in frequency of concomitant test reactions between the study population and an unselected test population in Gothenburg, Sweden.*

	Test-positive patients in the study population (number)	Test-positive patients in the study population (%)	Test positive patients in an unselected test population (%)	<i>P</i> -value
Colophonium	9	10	2.2	0.004
MPR	14	15	4.5	0.0002
FMI	12	13	7.1	0.021

Abbreviations: MPR, *Myroxylon pereirae* resin; FM I, Fragrance mix I

The differences in frequency of those concomitant reactions were even greater when beeswax-positive patients in the cohort and general test population were compared. Concomitant reactions to colophonium were observed in 29 % of the beeswax-positive patients (13 times more frequent, compared to an unselected test population), 53 % to MPR (12 times more frequent) and 29 % to FM I (four times more frequent).

Of the beeswax-positive patients, ten were also tested to caffeic acid (0.0005 – 5 %), CAPE and CADE (0.0001 – 1 %) in five different concentrations per substance. None of the ten reacted to caffeic acid, but three patients reacted to the esters, one to both in all concentrations, one to both in the two-to-three highest concentrations, and one to CADE in the highest concentration.

Using ToF-SIMS, caffeic acid and the two esters CADE and CAPE were detected in both white and yellow beeswax, with significantly higher contents ( $P < 0.05$ ) in yellow than in white. The relative surface content was higher of CADE than CAPE in yellow beeswax.

**Paper II:** In patch testing with propolis of four different geographical origins, 45 out of the 722 tested patients (6.2 %) reacted to at least one of the four propolis types from different geographical origins (Table 3).

*Table 3. Patients positive to propolis of four different geographical origins among 722 consecutive patients patch-tested in Gothenburg.*

Geographical origin	Test-positive patients, number (%)	95 % CI
China	26 (3.6)	2.4-5.2
North America	23 (3.2)	2.1-4.7
Sweden	16 (2.2)	1.3-3.5
Lithuania	22 (3.0)	2.0-4.5
Any origin	45 (6.2)	4.6-8.3

Abbreviations: CI, confidence interval

Propolis from China gave the highest number of positive reactions. Swedish propolis resulted in the lowest frequency of positive reactions. There were small differences among reactions to the propolis originating from the different countries, and those were not statistically significant. Reaction to only one of the propolis types appeared in 23 of the 45 positive patients (51 %), and the most frequent unique reaction was to the propolis from China, observed in 13 patients (29 %). Only six patients (13 %) reacted to all four types of propolis. Clinical relevance was indicated in 33 of the propolis-positive patients. Current relevance was recorded in nine (27 %) of those, meaning 20 % of all propolis-positive patients. Earlier relevance was recorded in two of the 33 propolis-positive (6 %) patients (4 % of all).

Of concomitant reactions among the propolis-positive patients to test substances related to plants and fragrances in the baseline series, the most frequent was MPR (43 %), followed by colophonium (23 %), and FM I (14 %) (Table 4). Those frequencies were significantly higher than in unselected dermatitis patients.

Table 4. Concomitant reactions between propolis and plant-related substances and fragrances in the Swedish baseline series in patients who tested positive (44<sup>a</sup>) and negative (677) to propolis.

	Patients with positive test results (number)	%	Patients with negative test results (number)	%	P-value
MPR	19	43.2 (6x)	51	7.5	<0.0001
Colophonium	10	22.7 (13x)	12	1.8	<0.0001
FM I	6	13.6 (2x)	40	5.9	0.019

Abbreviations: MPR, *Myroxylon pereirae* resin; FM I, Fragrance Mix I, x, times higher among propolis-positive patients compared to propolis-negative patients.

<sup>a</sup> the baseline series was not tested in one patient.

Doubtful reactions were found in 37 (5.1 %) patients, and irritant reactions in seven (1.0 %) patients. Two patients with late reactions were recorded, on D24 and D11 respectively.

**Paper III.** In patch testing with propolis of four different geographical origins in test centers in Denmark, Lithuania, and Spain, 54 of 1 470 tested patients reacted to at least one type of propolis (3.7 %), ranging from 1.3 – 5.8 %. As in paper II there were small, but not significant, differences between frequencies of reactions to the different origins of propolis, except when combining the results from the three countries, between propolis originating from Lithuania compared to Sweden ( $P= 0.039$ ). The frequency of positive reactions was higher in Lithuania than in Sweden. The most frequent unique positive reactions were to propolis originating from China and Lithuania (1.8 %). Reaction to only one of the propolis types was found in 29 patients (54 %). Reaction to propolis from China was the most frequent unique reaction and observed in 16 patients (30 %). Two patients (4 %) reacted to all four types of propolis. There were significantly higher frequencies of propolis-positive patients in Lithuania ( $P< 0.0001$ ) and Denmark ( $P=0.029$ ), compared to Spain. In 32 of the 54 test positive patients, clinical relevance was recorded, and current relevance was rated in 12 patients (38 % - meaning 22 % in all propolis-positive patients). Past relevance was recorded in 5 patients (16 % - 9 % of all). Concomitant reactions among the propolis-positive patients to fragrances or plant-related allergens in the baseline series were most common to MPR (33

%), followed by colophonium (31 %) and FMI I (22 %). Concomitant reactions to those allergens were significantly more common among propolis-positive patients, compared to propolis-negative, except for patients reacting to colophonium in Spain. Doubtful reactions to any of the four types of propolis were found in 76 (5.2 %) patients, all in Denmark. Irritant reactions to any propolis type were observed in eight (0.5 %) patients, also all from Denmark.

**Paper II + III.** When combining studies II and III, a total of 2 192 patients have been tested with the baseline series and propolis of four different geographical origins, the latter of the same batches in both studies. Out of those patients, in total 99 (4.5 % CI 3.7 – 5.5) patients reacted to any type of propolis (Table 5).

*Table 5. Number and frequency of positive patch test reactions to propolis of four different geographical origins, and total number and frequency of patients with positive reactions to any type of propolis tested.*

Test centers	Number of patients tested to propolis	Patients who tested positive to Chinese propolis	Patients who tested positive to North American propolis	Patients who tested positive to Swedish propolis	Patients who tested positive to Lithuanian propolis	Total number propolis-positive patients	CI 95 %
Denmark	448	9 (2.0%)	3 (0.7%)	5 (1.1%)	7 (1.6%)	16 (3.6%)	2.1-5.7
Lithuania	548	13 (2.4%)	14 (2.6%)	13 (2.4%)	16 (2.9%)	32 (5.8%)	4.1-8.1
Spain	474	5 (1.1%)	2 (0.4%)	0 (0 %)	3 (0.6%)	6 (1.3%)	0.5-2.6
Sweden	722	26 (3.6%)	23 (3.2%)	16 (2.2 %)	22 (3.0%)	45 (6.2%)	4.6-8.3
Total	2192	53 (2.4%)	42 (1.9%)	34 (1.5%)	48 (2.2%)	99 (4.5%)	3.7-5.5

Abbreviations: CI, confidence interval

The most frequent reactions were to the propolis of Chinese origin followed by the Lithuanian, the North American, and the Swedish propolis (Table 4). The only significant differences in all the four countries combined were more frequent positive patch test reactions to propolis originating from China, compared to propolis originating from Sweden ( $P=0.007$ ) and propolis originating from Lithuania and from Sweden ( $P=0.002$ ), with lower frequency in Sweden. Our results indicate that testing with only the Chinese propolis would have detected 53 % of all propolis-positive patients, and testing with only Lithuanian, North American, or Swedish propolis would have detected 48 %, 42 %, and 34 % of positive patients respectively.

Concomitant reactions to fragrance and plant extract markers of the baseline series among propolis-positive patients were most common to MPR (37 patients, 37.4 %), followed by colophonium (27 patients, 27.3 %), and FM I (19 patients, 19.2 %).

## 5 DISCUSSION

ACD is a widespread problem. It is especially distressing for the patient if very visible as on the lips or on the face (47). If an eczema is located on the lips and caused by something the individual topically uses to make the lips smooth, it can be especially problematic, as the symptoms are maintained or worsened by attempts to treat them. The detection of a CA is thus extra important in those cases.

The use of alternative or “natural” ways to maintain or treat the skin is gaining ever more interest in modern society (47). As beeswax and propolis are two such products, finding patients who are contact allergic to them is important. Knowledge of how to patch test with the right concentrations of both beeswax and propolis is therefore also important, as well as finding the right preparation of propolis, or the important hapten(s) of both products. Thereby it will be possible to identify as many individuals who have become sensitized as possible.

### 5.1 PAPER I - CONTACT ALLERGY TO BEESWAX

This is to my knowledge the first systematic study of CA to beeswax in Sweden. Hitherto beeswax has not been discussed as an important sensitizer in patients with cheilitis. One study from the United Kingdom published by Rajpara *et al.* of CA to beeswax in a general test population found that 0.45 % of study participants were beeswax positive (34).

We observed a high frequency, 18 %, of CA to white and/or yellow beeswax in patients with cheilitis or eczema of the face. The manufacturer of the lip balm, “Försvarets hudsalva” supplied us with yellow beeswax, which means that we tested with the same type of yellow beeswax that was used to produce the lip balm. This might partly explain the high frequency of CA to the yellow beeswax in the cohort. The high frequency of CA to beeswax in such patients is important as beeswax is very common in products used for the lips and for the face. In view of the widespread use of lip balms and lipstick, CA to beeswax must, however, be considered unusual. Considering our results it is surprising that a recent prospective study of 66 patients diagnosed with facial CD in Denmark identified only 1.5 % of the patients with CA to beeswax (47). It is not clear how many patients with contact cheilitis were included in this study, though.

The high frequency of concomitant reactions between beeswax and propolis indicates that the reactions to beeswax in fact may represent CA to propolis as an impurity of the beeswax. However, the high frequency of reactions to purified, white beeswax does not support this theory. CAPE and CADE, known to appear in propolis and considered important in causing propolis sensitization, were detected in beeswax, especially in yellow beeswax. This could indicate that the content of propolis in beeswax might be important from a sensitization perspective. However, only three out of ten beeswax-positive patients had a positive patch test to the two caffeates. Caffeates, considered to be the most important haptens in propolis, would be expected to be more frequently co-reacting with beeswax than we observed. On the other hand, not much is known about which proportion of propolis-allergic patients react to caffeates. There is one German study, by Hausen which has shown that 20 out of 27 (74 %) propolis-positive patients also reacted to CAPE and 17 (63 %) to CADE (95). In this study also many, 18, patients reacted to benzyl caffeate. No patients in this group tested positive to caffeic acid. We have not observed patients who patch-test positive to caffeic acid either. Previous reports have shown that there are some differences between CA to propolis, yellow beeswax and white beeswax (34, 73, 87). In an investigation of 2 828 consecutive dermatitis patients in the United Kingdom by Rajpara *et al.*, 55 (1.9 %) were patch-test positive to propolis (34). Only four (7.2 %) of these were patch-test positive also to beeswax. Thirteen patients (0.5 %) were test positive to beeswax, ten to white and three to yellow. A case report from Italy by Lucente *et al.* described a patient who was patch-test positive to bleached beeswax, but not to propolis (73). However, reports of concomitant reactions between yellow beeswax, white beeswax, and propolis have been published. Junghans *et al.* report a case in Germany of positive patch tests to beeswax and propolis in a patient reacting to a beeswax-containing ointment (71). Schuler and Frosch report a German study where two patients were positive to white beeswax, and two to yellow beeswax, out of four propolis-positive patients (103). Further investigations are required, both regarding the culprit of allergy in beeswax and evaluating how important the presence of propolis in beeswax may be. The results of our investigation must also be confirmed in further studies. If so, it shows that when investigating this type of patients, beeswax, propolis or the relevant hapten should be included in patch testing.

A stricter definition of the dermatitis localization would have been valuable in our study. Combining several localizations make it more difficult to assess the importance of the beeswax allergy. A more thorough chemical analysis of the two types of beeswax would have facilitated assessment of what may be the relevant hapten(s).

## 5.2 PAPER II AND III - CONTACT ALLERGY TO PROPOLIS

Study II is the first systematic study of CA to propolis in Swedish dermatitis patients. The high frequency of positive patch test reactions to propolis points towards its inclusion in the Swedish baseline series. The result must, however, first be confirmed in further studies in other parts of Sweden.

There are several previous studies in Europe showing frequencies of positive reactions of CA to propolis in general test populations, 0.5 – 7.6 % (74, 75, 82, 84), excluding one Polish study that differed substantially with a frequency of 15 % (75). The frequencies of CA to propolis in the studies in papers II and III, 1.3 – 6.2 % (Table 5), are in the same range as in the previous European studies, though in the upper part of those. In the previous European studies, patients are tested with only one commercially available propolis preparation. If the comparison is made using only one of the commercial types in our studies, the results are in the middle of the range. Our results and the decision to include propolis in the European baseline series from 2019 support the inclusion of propolis in the Swedish baseline series (84).

## 5.3 FREQUENCIES OF CONTACT ALLERGY TO PROPOLIS OF DIFFERENT GEOGRAPHICAL ORIGINS

Studies II and III are to my knowledge the first two studies of CA to propolis of different geographical origins in consecutive patients suspected of CD. The results point to that the differences in frequencies of positive patch test reactions are rather small. Two possible explanations of this are that the differences actually are rather small, or that the power is too low to demonstrate any differences. However, in our studies, just using one commercially available test preparation would have missed about half of propolis-allergic patients. To my knowledge there is only one study comparing CA to propolis of different geographical origins (92). In that study of beekeepers in Great Britain, a high percentage of concomitant reactions to propolis from different parts of the British Isles were present (84 %). The frequency of concomitant reactions to at least two of the different types of propolis in our studies II and III was 47 %. The difference in frequency might be due to bigger differences in composition between the propolis types in our studies, as they were from more geographical separated areas. The differences in frequencies of positive patch test reactions between the different propolis types are, however, rather

small also in our studies. When combining the results from studies II and III, there were no significant differences in frequencies of positive patch test reactions except for between propolis from China and Sweden ( $P = 0.007$ ) and from Lithuania and Sweden ( $P = 0.002$ ). In both cases, the frequency of positive reactions was less in patch testing with propolis from Sweden. The reason for this is not clear but might be that propolis from China and from Lithuania are more common in consumers products than propolis from Sweden. The result might be different in an investigation of beekeepers from Sweden, who would be expected to be more prone to come in contact with Swedish propolis, from their own beehives.

Power is low (12 - 60 %) in the comparison of differences in frequencies of patients positive to propolis of two different origins, in all the four countries, except for propolis from Lithuania compared to propolis from Sweden (99 %), and Chinese propolis compared to Swedish (85 %) when assuming that the exact proportions are the estimated proportions. This is mainly due to low number of tested patients and small differences in frequencies.

One weakness of both studies II and III is the lack of information of which plants were growing in the area around the beehives from where the propolis was collected. This is a challenging problem to overcome as it would mean a botanical investigation around each hive from which propolis was collected at the time of collection. Information on the botanical surroundings of the beehives was likewise not known for the propolis in consumer products. Another weakness is that detailed analysis of chemical differences between the four types of propolis is lacking. We have not had access to such analysis methods. A high proportion of the propolis-positive patients was not assessed regarding clinical relevance. A higher proportion assessed for clinical relevance would have been desirable, but it is hard to accomplish this with many different patch test readers.

#### 5.4 CONCOMITANT REACTIONS BETWEEN BEESWAX, PROPOLIS, AND PLANT-DERIVED, OR FRAGRANCE PREPARATIONS FROM THE BASELINE SERIES

High frequencies of concomitant reactions between both beeswax and propolis compared to MPR, colophonium, and FM I have been found. This should be kept in mind when discussing products to avoid with patients who are contact allergic to any of these. The reason for concomitant reactions can be any of

true cross reaction, metabolic transformation of haptens, co-sensitization, or a combination of those. These high frequencies may be one explanation for why the frequency of clinical relevance was not higher than observed. Some patients who patch-test positive to propolis might never have been in contact with propolis but have rather been sensitized by a plant substance or a fragrance. It is generally difficult both for the patient and the doctor to identify all possible exposures. The three studies confirm that there is a high frequency of concomitant reactions between bee products and plant-related test preparations and fragrances. The results from our studies show concomitant reactions between propolis and MPR in the same range, 38 % compared to 13 - 90 % in previous studies, though the previous studies have shown very varying results (33, 34). Those concomitant reactions might to a certain degree be due to common haptens like benzoic acid, benzyl benzoate, cinnamyl alcohol and cinnamal cinnamate (33). The frequencies of concomitant reactions between propolis and FM I and colophonium in our studies were in the same range as previous studies, 19 % compared to 11 - 25 % (30, 34) and 28 % compared to 13 - 27, respectively (30, 34). Propolis and colophonium share among other haptens, abietic acid (34). Shi *et al.* have in an American study shown that it is not necessary for patients who are contact allergic to fragrances to avoid propolis, as many of the haptens in fragrances are not present in propolis (30). Patients who are contact allergic to propolis should on the other hand avoid contact with fragrances due to common haptens like cinnamyl alcohol and eugenol.

## 5.5 PATCH TEST CONCENTRATIONS OF BEESWAX AND PROPOLIS

The previously recommended patch test concentration for beeswax is 30 % pet (104), but also a concentration of 20 % has been used (72). This is, however, hard to manufacture without heating, which might change the beeswax chemically. We have thus chosen to use beeswax 100 % which has also been used before without problems described (47, 72). As there were no irritant reactions and just one doubtful to each of white and yellow beeswax, we had no indications that the test concentration was too high.

Propolis is generally patch-tested in 10 % pet, which is the concentration used in our studies (34, 47, 75). In one Italian study by Giusti *et al.* among 1 255 consecutive children with dermatitis, propolis 20 % pet was used. In this study, 5.9 % of the patients were patch-test positive to propolis. Four doubtful reactions (0.03 %) and no irritant reactions were reported (61). In a study by

Barile *et al.* also from Italy, propolis 20 % pet was used in 305 consecutive patients with psoriasis, 96 consecutive patients without psoriasis or dermatitis, and 743 dermatitis patients without psoriasis. There were no reports of doubtful or irritant reactions (85). Schena *et al.* has reported patch tests of 42 cheilitis patients tested with propolis “as is” without any report of doubtful or irritant reactions (52). No active sensitization was reported in any of these three studies. Animal tests on guinea pigs have been carried out, and these did not show a high frequency of irritancy by propolis when patch-tested in 20 % pet (105). The frequencies of doubtful reactions to propolis in our studies varied between different clinics but were generally high. One explanation for the high frequency of doubtful reactions could be that the normally used test concentration of 10 % is too low. Some of the doubtful reactions might be weak positives, which might be clearly positive at a higher test concentration. This issue has been discussed by other authors, both regarding propolis (74) and regarding for example methyl dibromo glutaronitrile (106). Biased subjectivity and regional differences in the readings of doubtful and irritant reactions is something to take in mind and a problem in all multicenter studies. The low frequency of irritant reactions in our studies could, however, be an indication that a higher test concentration could be used. The issue should be investigated further, taking in mind the risk of active sensitization. Which is the optimal patch test concentration and test material for propolis are questions raised by studies II and III. High patch test concentrations of complex products like propolis or MPR (generally patch-tested in 25 % pet) do not necessarily mean that the test concentration of the causative hapten is high. The content of the hapten in the product might be low and diluted further as the product is diluted. It has also been reported that a test concentration that is too high can increase the number of doubtful reactions (107). Sometimes it can be difficult to distinguish between weak positive, doubtful, and weak irritative test reactions and this can be prone to subjectivity. As a matter of fact, a doubtful test reaction is either weak positive or weak irritant, but not enough to be considered as either one. This uncertainty is a weakness of studies II and III, as in most other patch test studies (26, 108). The problem is, however, hard to overcome. One solution is calibration between individuals reading tests, though this is time-consuming and labour-intensive (109). To determine the optimal test concentration, patch tests can be performed with dilution series. This becomes, however, complicated when performed on a large scale. A more practical way might be to compare two different concentrations and assess the frequencies of positive, doubtful and irritative test reactions. Efforts have also been made to find differences in histology and cytokine expression to facilitate the assessment of test reactions (9). Those are, however, not in practical use yet. Guidelines from, for example, the ESCD (25) are helpful for standardizing patch test readings. Results from investigations of patch testing, especially

multicenter studies, though the best tool we have for diagnosing CA must however be assessed with caution (26, 110).

The two late reactions in study II are according to current guidelines active sensitization (25). It can be argued though that they were simply late reactions due to low test concentration. There are examples pointing towards this in the literature regarding other test substances (111-113). Hagvall *et al.* found in a study of epoxides of cinnamyl alcohol, present in FM I, a total of five patients with late positive patch test reactions. Two of those had a doubtful reaction to FM I and one was positive to FM I at the regular reading time (111). Engfeldt *et al.* reported a case of a patient with known CA to palladium who, at a two-years follow-up retesting, had a late reaction on D11 (112). Isaksson *et al.* reported one patient reacting to hydroxyethyl methacrylate on D10 after having been doubtful at the regular reading time (113).

## 5.6 TOF-SIMS

The ToF-SIMS method has been used in different fields due to its ability for surface characterization without changing the sample investigated. For example, it has been used in different applications such as in characterization of the biomaterial surface chemistry of implants, of probing the chemistry of the bacterial envelope of a microorganism, and for imaging fingerprints chemically on banknotes (45, 114, 115). To the best of my knowledge, this is the first investigation using ToF-SIMS to detect haptens on the surface of a solid material.

## 6 CONCLUSION

CA to beeswax is common among patients with eczematous cheilitis. The caffeates, CAPE and CADE, are detected in beeswax, indicating presence of propolis.

CA to propolis is so common in Swedish patients with CD that its inclusion in the Swedish baseline series should be considered.

Although there are small, and just a few significant, differences in tendency to result in CA between propolis from China, North America, Sweden, and Lithuania, it is important to note that using only one of the commercial test preparations of propolis identifies just about 50 % of all propolis-allergic patients.

Concomitant patch test reactions between beeswax and propolis are common, and so are concomitant reactions between both of those and colophonium, MPR and FM I.

## 7 FUTURE PERSPECTIVES

The results from this thesis may point to further patch test investigations regarding propolis of different concentrations to determine which is the optimal test concentration. The frequency of CA to propolis in Swedish patients with CD must also be confirmed by further studies in different Swedish test centers. To better understand the importance of CA to a person's own, local propolis, patch testing Swedish beekeepers with propolis from Sweden, their own beehives, and other geographical origins would be of interest. Likewise patch testing in different parts of Sweden with propolis from different areas in Sweden would be of interest to investigate possible regional differences, as has been done in Great Britain. To find the optimal patch test substance when investigating patients for suspected CA to propolis and beeswax, it would be of specific value to patch test patients allergic to propolis and beeswax with oxidation products of caffeates, and other suspected haptens in propolis, and beeswax. Further chemical investigations like analysis of oxidation products from CAPE and CADE and their allergenic potential as well as further investigations of possible haptens in beeswax would shed more light on those complex issues.

## ACKNOWLEDGEMENTS

There are many to whom I owe a lot of gratitude for helping with, participating in, and guiding in writing this thesis.

First and foremost, my main supervisor Lina Hagvall for her enormous patience, kindness, and enthusiasm through all discussions, and also for pushing onwards when needed.

My co-supervisor Amra Osmančević, for encouraging comments, dear friendship, and constructive criticism.

All my co-writers for their help, input, and clever comments: Annika Inerot, Mimmi Tang and Per Malmberg, Gothenburg; Sara Oldberg Wagner, Borås; Katarzyna Prystupa-Chalkidis, Skövde; Kristina Ryberg, Uddevalla; Ana Maria Giménez-Arnau, Barcelona; Jūratė Grigaitienė and Laura Malinauskienė, Vilnius; and especially Evy Paulsen, Odense for her many comments and advice.

Magnus Bruze, Malmö, for always taking of his time for invaluable discussions and insights.

Anette Gente-Lidholm and Sam Polesie, Gothenburg, for practical advice in producing a thesis, and always being helpful.

Martin Gillstedt, Gothenburg, for help with the statistics.

Britt-Marie Ehn, Gothenburg, for help with the logistics, preparing test material, friendship and encouraging support.

Masoumeh Dowlatshahi Pour, Gothenburg, for showing me the wonders of ToF-SIMS.

Aistė Beliauskienė, Kaunas, for logistical help with Lithuanian propolis, lending her hand for the photo of raw propolis, and overall being such a nice person.

Gunilla Färm, Stockholm, who except being a wonderful friend was the first to get me interested in contact dermatitis.

The patch test personnel in Borås, Skövde, Uddevalla, Barcelona, Odense, and Vilnius.

My dear friends and colleagues at Hudläkarmottagningen Telegrafan for being such wonderful comrades at work.

Colleagues and friends at the Department of Dermatology, Gothenburg, for making me feel younger.

All friends and colleagues at the Malmö Occupational and Environmental Dermatology Clinic as well as in the Swedish Society for Occupational and Environmental Dermatology and the Swedish Contact Dermatitis Group for interesting discussions and nice togetherness.

My sons, Anders, and Björn for being there and my beloved wife, Katarina, for putting up with me throughout it all.

The financial support from the Gothenburg Society for Medicine, from "Hudfonden" Sweden, and from the Local Research & Development Council of Södra Älvsborg are much appreciated.

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